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The expression of different cytoplasmic domains could influence the function of the α6β1 laminin receptor integrin by affecting the ligand binding characteristics of the integrin or by altering the intracellular interactions between α6 and cytosolic components (i.e.- cytoskeleton or signaling molecules) thereby mediating unique cellular responses as a result of adhesion to the extracellular matrix. The goal of this proposal is to dissect the biological roles of the alternatively spliced cytoplasmic domains of α6. To accomplish this I have established a panel of ES cells expressing human α6A and α6B. The ES-α6A cells exhibit a unique morphology, characterized by the presence of cytoplasmic extensions and lamellipodia. In contrast, the ES-α6B transfectants have the same morphology as wild type ES cells, growing in smooth-bordered islands. ES-α6A cells showed a 10-fold increase in migration towards laminin, compared to ES and ESα6B cells. When assayed using video microscopic techniques, ES-α6A cells exhibited classical lamellipodia movement and are more motile than ES and ESα6B cells. This suggests that the α6A cytoplasmic domain interfaces with cytoplasmic molecules that direct migration.

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INTRODUCTION

Integrins are a widely expressed family of cell surface receptors (Hynes, 1987). This diverse family of transmembrane glycoproteins appears to be the major receptors by which cells attach to the ECM, and several integrins also mediate cell-cell interactions. Integrins are expressed as non-covalently associated heterodimers consisting of an α and a β subunit. The integrin extracellular domains form a ligand-binding site recognizing one or more extracellular ligands or counter-receptors on other cells. To date, in vertebrates, 14 α and 8 β subunits have been identified. Although these could combine to produce more that 100 heterodimers, the actual diversity is much more restricted and currently only 20 different receptors have been identified. Many α subunits associate with only one β subunit, however, there are a few exceptions, such as α4, α6, and αv, which associate with more than one β subunit resulting in more diverse ligand associations. Additionally there are several instances of alternatively spliced domains existing in α3, α6, α7 and β4 (Hynes, 1992; Collo et al., 1993). The potential for diversity in this receptor family is therefore quite large.

Integrins play a fundamental role during the entire life of an organism. As one example, early in embryo development, fibronectin receptors are thought to play a crucial role in mesoderm migration (Elices et al., 1991). In fact when the α5 or α4 subunits are disrupted, mouse embryos die early in gestation (Yang et al., 1995; Yang et al., 1993). In normal adult physiology, integrins play varied roles, from the establishment of hemidesmosomes in keratinocytes to facilitate wound healing (Kurpakus et al., 1991), to the binding of soluble fibronectin by platelets to initiate clot formation (Hynes, 1987). Additionally, integrins may play a role in disease states, such as cancer, where modulation of integrin expression may play a crucial role in metastasis (Mareel et al., 1992).

Cell adhesion plays a key role in tumor progression and metastasis. In order to invade and metastasize, tumor cells must acquire the ability to detach from the primary site, cross basement membranes, migrate, traverse vessel walls and reattach in a new site. Cell adhesion molecules, such as integrins, must play key roles in all of these steps. Recent publications have suggested that integrins may be considered tumor associated antigens given their apparent deregulation in various types of tumors (Miettinen et al., 1993). Altered expression or function of integrins may directly influence the ability of a tumor cell to detect basement membrane boundaries and/or confer novel attachment and invasive properties.

A substantial body of literature currently documents altered or deregulated integrin expression in tumor cells (Koukoulis et al., 1991;

Carey et al., 1992; Lin et al., 1993; Ruiz et al., 1993; Sager et al., 1993). The integrins α6β1 and α6β4 bind isoforms of the extracellular matrix protein laminin and exhibit altered expression patterns in tumor cells as compared to normal tissue. In particular, altered patterns of α6 integrins expression have been documented in breast cancer (Koukoulis et al., 1991), squamous cell cancer of the head and neck (Carey et al., 1992), and fibrosarcomas (Lin et al., 1993). In separate studies, antibodies specific for α6 suppressed mouse melanoma metastasis (Ruiz et al., 1993). Additionally, α6 has been identified as a potential tumor suppresser gene. Using the differential display method of differential expression cloning, Sager et al. (Sager et al., 1993) detected loss of α6 expression in metastatic breast tumor cells and low expression in primary breast tumor cells, compared to normal mammary epithelial cells. Concurrent with α6 as a tumor suppresser, Gui et al. (Gui et al., 1995) show that loss of α6 expression correlates with the presence of axillary metastasis in breast cancer. In direct opposition, however, Freidrichs et al. (Friedrichs et al., 1995) show that high expression of α6 is correlated with reduced survival of breast cancer patients. Although all of these results are compelling, a simple overview of these and other current data, however, establishes that there is no consistent trend towards gain or loss of α6 integrins expression and tumor cell metastasis or cancer progression. Instead, it is clear that we must understand the function of α6 integrins before we can define their role in cancer progression. The studies supported by this proposal will dissect the specific contributions of the α6A and α6 B isoforms to integrin function, will further define the mechanisms by which cells respond to the ECM, and ultimately will help interpret the relevance of altered integrin expression on tumor cells. This information could be used to develop rational therapeutics for breast cancer treatments.

Just as the extracellular domain mediates specific matrix contacts, the short (less than 50 amino acids) cytoplasmic domains of integrins have been shown to mediate specific intracellular interactions. Integrins function as a transmembrane link between the ECM and the interior of the cell. There is evidence that some integrins associate with components of the microfilament system, possibly through a connection with talin, vinculin, or a-actinin. This interaction results in the localization of integrins, *in vitro*, in focal adhesion contacts (Burridge et al., 1988). More specifically, deletion of all or part of the β1 cytoplasmic domain interferes with integrin associations in focal contacts (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990) Evidence of α subunit interactions with the cytoskeleton is less direct, however the unique cytoplasmic domains of α subunits appear to direct cytoskeleton associations and the cytoplasmic domains of different integrins may

mediate different contacts. This has been demonstrated in the example where α5β1 localizes to focal adhesion contacts, while α3 β1 does not, although both are fibronectin receptors and share identical β subunits (Elices et al., 1991). Additionally, different α subunit cytoplasmic domains have been shown to mediate distinct cellular functions such as collagen gel contraction or cell migration (Chan et al., 1992). Clearly, given the diversity among subunit cytoplasmic domains and the existence of alternatively spliced cytoplasmic domains (Tamura et al., 1991; Cooper et al., 1991), multiple opportunities are possible for a subunits to direct unique cellular responses to identical ECM ligands.

The α6 integrin subunit can associate with one of two β subunits, β1 and β4 (Tamura et al., 1991; Hemler et al., 1988). The α6β1 heterodimer has been shown to function as a laminin receptor (Cooper et al., 1991; Sonnenberg et al., 1988) and is expressed on cells of myeloid, lymphoid, and epithelial origin, as well as other cell types (Sonnenberg et al., 1990). The α6β4 heterodimer is expressed in a more restricted pattern. It is detected on various epithelial cells, endothelia, and peripheral nerves (Jones et al., 1991; Quaranta and Jones, 1991). Recent data suggest that α6 integrins may mediate cell-cell interactions in addition to cell-ECM binding. The monoclonal antibody EA-1, which specifically recognizes α6, has been shown to block melanoma cell binding to endothelial cells (Ruiz et al., 1993). To date, the cellular ligand is unknown, and it is possible that cell-cell interactions are the result of activation of the α6 integrin.

Two variants of the α6 integrin subunit, α6A and α6B, have been identified in Dr. Quaranta's laboratory (Cooper et al., 1991; Tamura et al., 1991). These variants differ in the structure of their cytoplasmic tails and appear to arise from alternative mRNA splicing. The cytoplasmic domain of α6A is 36 amino acids in length, the cytoplasmic domain of α6B is 54 amino acids in length and the two domains share little homology. This proposal represents an approach to define the functional roles of the alternatively spliced cytoplasmic domains of the integrin subunit α6.

Mouse embryonic stem (ES) cells express only α6B in the undifferentiated state, then begin to express α6A upon differentiation (Cooper et al., 1991). We extended studies on this developmental regulation of α6 isoform expression by following α6A expression in the developing mouse embryo (Collo et al., 1995). Using PCR and immunohistochemistry, we determined that the embryo exclusively expresses α6B until 8 days of gestation. At this point, α6A is only expressed in the developing heart. As the heart develops, α6A is expressed in a gradient in the myocardium. α6A expression parallels the development of trabeculae in the heart. We postulated that α6A may mediate a more migratory phenotype, as it appears that these cells are migrating towards the inner chambers of the developing heart tube. This

distinct regulation of expression suggests that the A and B isoforms may be exploited by different cell types to regulate the cellular response to laminin binding.

The regulation of adhesiveness of the α6 integrin may be intrinsic, in that the conformation of the cytoplasmic domain directly alters the binding affinity of the extracellular domain. An additional possibility, however, is that the different cytoplasmic domains of α6 interact with unique subsets of cytoplasmic adapter molecules that regulate adhesiveness of the receptor. The diversity of response could be expanded further if the adapter molecules are cell-type specific or developmentally regulated to coordinately influence the function of the integrin. The expression of alternative cytoplasmic domains also may function to couple identical adhesion events to unique cellular responses. In this regard, subunit and cell-type specific cytoplasmic adapter proteins may couple adhesion to distinct cellular responses, thereby increasing functional flexibility in response to binding an identical ECM component. I have begun to address these hypotheses with the model system presented in this proposal. I have established a panel of ES cells expressing human α6A and α6B. Although there is no detectable difference in adhesion to laminin, the ES-α6A cells exhibit a unique morphology, with more cytoplasmic extensions, and the ES-α6A cells show a 10-fold increase in migration towards laminin. This suggests that the α6A cytoplasmic domain interfaces with cytoplasmic molecules that direct migration.

BODY

When developing a model system to study the function of the α6A and α6B tails, it would be helpful to utilize a cell line for transfection that does not express the α6 integrin. However, since we hypothesize that the cytoplasmic tails interact with cytoplasmic factors that may link the integrin to downstream events, we would like to use a cell line that contains all the necessary "co-factors". Therefore, the approach I have taken is to selectively delete the α6 gene product from a cell that normally expresses the integrin. Unlike the approach taken by Shaw and Mercurio (Shaw and Mercurio, 1994) and Delwel et al. (Delwel et al., 1993), using a mutated ES cell line will allow for reconstitution with various α6 constructs in a "normal" background. Indeed, since differentiated ES cells normally switch to express α6A, I will be able to probe for both α6B specific and α6A specific effector molecules simply by allowing the transfected cells to differentiate.

Substantial effort has been expended toward the goal of producing an α6 null ES cell, as detailed in Aim 2 of this proposal. The vector p244 shown in Fig 1 has been used for transfection into ES cells using electroporation. Briefly, $1x10^7$ cells were washed with electroporation buffer. Linearized DNA (20 μg) was mixed with the cells on ice, then the cells were subjected to electroporation using a BioRad gene pulser, 0.5 kvolt, 250 μFarads. After 10 min on ice, the cells were transferred to plating media. Twenty-four hours later, cells were selected in media containing 300 μg/ml G418 and 2 μM gancyclovir. Approximately 14 days post transfection, single colonies were picked and disaggregated in trypsin. Duplicate plates were grown. One plate was harvested to prepare genomic DNA for screening by PCR using the methods described by Laird et al. (Laird et al., 1991). PCR was performed on DNA prepared from pools of 8 clones using primer C in the neo gene and primer B in the native α6 gene (Fig 1A). The PCR products were run on an agarose gel, transferred to nylon, and probed with oligo E that had been end labeled with ^{32}P. This procedure identified positive PCR products in reactions using p209 as a control (Fig 1B). A total of 7 transfections were performed and 1852 colonies were screened using this procedure. Although 5 potential "light" positives were identified, when they were expanded and analyzed as single clones, all were negative for homologous recombination. As stated in my proposal, since this vector did not yield cells with targeted mutations within a reasonable time frame, I have begun construction of a new targeting vector that will be created with DNA isogenic to the ES cells and will be designed to disrupt the exon containing the ATG of α6. To that

end, I have screened a 129 mouse genomic library (Stratagene) and I have characterized several clones at the DNA level. One clone has been subcloned and is being screened by sequencing to define the intron/exon boundaries that are present. I anticipate having a new targeting vector by October, 1995.

In accordance with Aim 1, I have established a quantitative adhesion assay using the ES-1 cells. This assay was performed essentially as described by Calof and Lander (Calof and Lander, 1991) and represents a major technical departure from standard adhesion assays as described by Cooper et al. (Cooper et al., 1991) or Shaw et al., (Shaw et al., 1993), which are used predominantly in the field. The most prominent advantages are as follows: 1. Contact between the cells and the substrata is synchronized by centrifugation. This is relevant, since cells may settle at varying rates when simple gravitational sedimentation is employed. Additionally, centrifugation decreases the incubation time, which is important when analysis is performed on cells that are capable of depositing their own matrix. 2. The geometry of the custom assay plate decreases the occurrence of background due to non-receptor mediated trapping in the edges of the flat-bottomed wells usually employed. In the proposed assay, the gasket creating the cell wells is removed before samples are quantified, thereby removing the potential for artifacts. 3. Centrifugation is used to remove non-adherent cells. This technique potentially allows the added dimension of varying centrifugation force to assay degrees of binding as compared to standard assays, which simply wash away non-adherent cells with buffer. 4. Washing steps are replaced with immersion of the plate in buffer, thereby eliminating inconsistencies in washing conditions.

An example of the optimized assay is shown in Fig 2. The wells of the assay plate were coated with a titration of EHS laminin or bovine fibronectin, incubated at 37°C for 4 hrs, washed, and blocked with a BSA solution. ^{35}S-methionine-labeled ES cells were introduced into the wells at a concentration of 50,000 cells/well. The plates were sealed and immediately centrifuged at 600 rpm to synchronize cell contact with the substratum. The cells were allowed to bind for 30 min at 37°C, then the plates were flooded with warm PBS, sealed, inverted and centrifuged for 8 minutes at 800 rpm. The entire plate, still inverted, was submerged in cold PBS and then in fixative (3.7% formaldehyde / 5% sucrose / 0.1% Triton X 100 /PBS). After air-drying, the bound radioactivity, representing cell adhesion, was quantified on a Molecular Dynamics phosphorimager. Non-specific background was measured by cell binding to BSA coated plates, and was essentially zero. The laminin concentration chosen for all future experiments was 40 μg/ml.

Since α6 null ES cells were not available yet, preliminary

transfection experiments were begun using wildtype cells. Full length α6A or α6B was subcloned into the expression vector pBJneo. α6 expression was driven by the SRα promoter (Takebe et al., 1988), composed of the SV40 early promoter and the R-U5 region from the long terminal repeat of human T-cell leukemia virus type 1. Electroporation conditions were performed as described above. After approximately 14 days of selection in G418, colonies were harvested in bulk, expanded, and cells were prepared for fluorescence activated cell sorting (FACS). The monoclonal antibody BQ16 was used to detect transfectants. BQ16 specifically recognizes human α6, with no binding to untransfected, mouse, ES-1 cells. Positive cells were sorted, expanded, and further experiments were performed on the bulk positive population. Fig 3 shows that the two cell lines chosen for analysis, ES6A.5 and ES6B.1, express similar levels of human α6, as detected by BQ16 binding. As control, ES-1 cells exhibit no binding to BQ16.

To further characterize the transfectants, detergent lysates were prepared for western analysis. Briefly, cells were trypsinized, blocked with trypsin inhibitor, washed, and lysed in 2% Renex in PBS with 0.174 μg/ml PMSF, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin. Lysates were incubated on ice for 1 hr, then centrifuged at 40,000 rpm for 1 hr. Lysates were separated on 4-20% SDS-PAGE gradient gels (Novex, San Diego, CA) under reducing and non-reducing conditions and then transferred to PVDF (Millipore, Bedford, MA). Expression of the transfected α6A was detected using a polyclonal antibody raised against the cytoplasmic tail of α6A (6845) which has been affinity purified on an α6A peptide column (Collo et al., 1995). The 6845 antibody does not detect any protein in untransfected ES-1 cells (Cooper et al., 1991), therefore the 140 kD band detected in Fig. 4 is human α6A. Similar western blots were performed on ES6B.1 using a polyclonal antibody against the α6B cytoplasmic tail. There was significant background with this antibody (data not shown) and I am currently performing affinity purification to optimize the results.

The initial question addressed, using the transfected cells, was whether adhesion to laminin was altered when α6A was expressed in a cell that normally expressed α6B. Since ES-1 cells normally expressed α6B β1, it was not expected that we could assay differences in adhesion mediated solely by the α6A or α6B molecules. Indeed, as shown in Fig 5, both ES6A.5 and ES6B.1 cells bind EHS laminin to the same degree as ES-1 cells. These studies are not complete. It is possible that expression of α6A will alter the degree of adhesiveness. To address this, I will take advantage of the design of this adhesion assay and subject the panel of cells to different G forces, by increasing the rpm used to remove nonadherent cells after adhesion. This will determine whether α6A and

α6B contribute differently to the "strength of adhesion". I am also initiating experiments to compare cell adhesion to isoforms of laminin. Our lab has purified and is characterizing the laminin isoform, laminin 5r (α3, β3, γ2),. Therefore I will extend these studies to include laminin 5r. Fig 5 shows a preliminary experiment in which ES-1 cells bind laminin 5r. I will continue this work and assay the transfected cells for 1) adhesion and 2.) strength of adhesion.

ES-1 cells are a subline isolated by Dr. Helen Cooper (Cooper et al., 1991) by growing D3 ES cells without a fibroblast feeder layer in the presence of leukemic inhibitory factor (LIF) on tissue culture plates coated with denatured gelatin. In this system, ES-1 cells exhibit the same morphology as other ES lines. In particular, the cells grow in islands and have very smooth borders. During normal passage, it became evident that the ES6A.5 cells had a significantly different morphology. Unlike the ES-1 and ES6B.1 cells, ES6A.5 cells had many cytoplasmic extensions and did not maintain small islands of smooth colonies. Fig 6 shows a comparison of ES-1, ES6B.1 and ES6A.5 cells grown on gelatin. The same differences in morphology were also seen when the cells were grown on laminin.

As proposed in Aim 1, it was possible that the α6A and α6B isoforms confer different levels of motility to the cell. To address this question, two types of migration assays were performed. Preliminary experiments monitored cell motility on glass coverslips coated with EHS laminin. ES-1, ES6A.5 or ES6B.1 cells were centrifuged onto coated coverslips and immediately transferred to a microscope with a heated stage equipped with video-tracking equipment. A field of cells was monitored for 2 hrs. Analysis of the condensed 2 hrs showed that the ES6A.5 cells extended more pseudopodia and moved approximately 3 microns, compared to ES-1 and ES6B.1 cells which moved only approximately 1 micron (data not shown). These experiments will be expanded further to cover longer times (8hrs) to directly quantify the distance migrated for all cell types.

The second migration assay performed utilized Transwell chambers (Costar, Cambridge, MA). These chambers have polycarbonate filters with 8 micron pores. Matrix molecules were coated on the bottom side of the filters and cells were plated on the upper side of the filters. After an 18 hr incubation at 37°C, the cells on the membrane were fixed and the top of the membrane was swabbed to remove cells that had not migrated towards the matrix. For quantitation, the filters were cut out of the chamber, mounted on slides, and stained cells were counted using a 40x objective. Fig 7 shows that ES6A.5 cells exhibit significantly higher migration towards EHS laminin than ES-1 or ES6B.1.

Interestingly, ES6A.5 cells also migrated well on fibronectin. Several explanations could address this result. An intriguing possibility

was that expression of α6A cross-activated the fibronectin receptor. If this were the case, the migration would be inhibited by the peptide "RGD". It has been clearly shown that "RGD" disrupts integrin binding to fibronectin (Cheresh, 1991). To address this hypothesis, the transwell assay was performed in the presence and absence of 268 μM RGD peptide (Barker et al., 1992). As a control for RGD inhibition, M21 cells (Felding-Habermann et al., 1992) were included. Fig 8 shows that RGD did not significantly inhibit ES6A.5 migration towards fibronectin, while the M21 cells were completely inhibited. This suggested that the ES6A.5 cells were not using their fibronectin receptor to migrate through the filter. A more likely explanation for the increase in ES6A.5 migration on fibronectin was that the ES6A.5 cells deposited their own laminin and used this newly synthesized matrix for migration. A direct test of this will be to block the migration with an antibody directed against α6. The experiments using GoH3 (Sonnenberg et al., 1986) to block α6 mediated migration are currently being performed. In support of the hypothesis that cells are migrating on matrix they are secreting, Fig 9 shows that ES6A.5 cells migrate to a small degree on membranes coated with gelatin or membranes simply incubated with HBSS (data not shown). Experiments are currently being performed to directly assay for laminin deposited by the cells using western blots and immunofluorescence. Comparison of the assays using gelatin or uncoated membranes for migration to migration on fibronectin showed that ES6A.5 cells still migrated to a greater degree if fibronectin is present. Future experiments will survey other matrices to determine which proteins support increased migration. Interpreting the mechanism of increased migration will be pursued over the next several months. The exciting preliminary conclusion of this work is that the expression of α6A in a cell that normally expresses α6B confers a novel migratory phenotype. This is an important milestone in defining the functional differences between the α6A and α6B cytoplasmic tails.

CONCLUSIONS

In summary, using ES cells transfected with human α6A or α6B, I have shown that the α6A integrin subunit conferred a migratory phenotype. This was shown both by tracking motility on laminin-coated coverslips with video microscopy and also by cell migration through a membrane towards laminin. Additionally, the ES6A.5 cells exhibit a novel morphology. The cells have more cytoplasmic extensions and extend lamellipodia compared to untransfected ES-1 cells and ES6B.1 cells. This is an important finding towards the goal of this proposal of understanding the functional differences between the α6A and α6B cytoplasmic tails of the α6β1 laminin receptor.

This finding is of great importance to the continuation of this grant at several levels. First, it shows that significant findings can be generated using wild type ES cells as a model system to analyze the function of the alternative cytoplasmic domains of the α6 integrin. Although this is true, these findings may be extended further if the α6-null cell is available. For that reason, I plan to continue my efforts towards generating the α6-null ES cell line. Second, the current findings will serve as an assay by which I can analyze the effect of mutations imposed on the α6 cytoplasmic domain. Aim 3 of the original proposal outlined a series of experiments designed to map regions of the cytoplasmic tail that confer particular functions to the integrin. Using migration as a test for gain/loss of function, I may be able to define specific amino acid sequences that are required for migration. Finally, this panel of cell lines will open the door for additional experiments that were not originally proposed, but would expand our understanding of the function of the cytoplasmic domains of α6.

The new experiments I plan to perform are designed to address the question of whether the cytoplasmic domains interact with cytoplasmic molecules and if there are differences between the molecules that interact with α6A or α6B. Recent data have expanded the view of the function of integrin receptors. In addition to serving as adhesion molecules and contacts to cytoskeleton, integrins have been shown to function as signaling receptors that intersect with other signaling pathways in the cell (Schwartz, 1992; Juliano and Haskill, 1993). After engagement with ligand, integrins transduce signals that affect gene expression, tyrosine phosphorylation, and levels of intracellular pH or calcium (Schwartz, 1992). The biochemical pathways and specific second messenger molecules involved in this signal transduction are just beginning to be elucidated, however one prominent association has been documented between the platelet integrin α IIb β 3 and focal adhesion kinase ($pp125^{FAK}$) (Lipfert et al., 1992; Zachary and Rozengurt, 1992). In fact, prelimnary evidence suggests that α6 interacts with signaling

molecules. Gimond et al. (Gimond et al., 1995) showed that the α6A tail can be phosphorylated *in vitro* by protein kinase C. Additionally, de Curtis and Gatti (de Curtis and Gatti, 1994) showed that α6 can be isolated in a large complex on sucrose gradients, although the proteins associated have not been characterized. I propose to use the methods of Plopper et al. (Plopper et al., 1995) to isolate α6A and α6B integrin associated complexes after engagement of the receptor by EHS laminin, laminin 5r or anti-human α6 antibodies. We will perform *in vitro* kinase assays to determine if any kinase molecules are associated with the complexes. Additionally we will perform western blot analysis to probe for the presence of known signaling molecules in the complex. The hypothesis is that there will be some known molecules associated with the integrin complex and that this profile may be different between the α6A and α6B isoforms.

In summary, during the first year of funding for this grant, significant progress has been made towards the achievement of all 4 aims. Although the α6-null cell has not been obtained, new efforts are being designed and implemented to obtain that cell line. The quantitative adhesion and migration assays have been optimized for ES cells. Most importantly, a panel of transfected cell lines has been created using wild type ES cells as a host. Although the parental cells express mouse α6B, a novel finding, namely that α6A confers a migratory phenotype on ES cells, has been described. This finding has validated the use of this model system and will allow the pursuit of aims 3 and 4, as well as experiments designed to identify cytoplasmic molecules interacting with the α6A and α6B cytoplasmic domains. Understanding the function of the α6 integrin subunit will be of paramount importance to our understanding of development and in the understanding of carcinogenesis.

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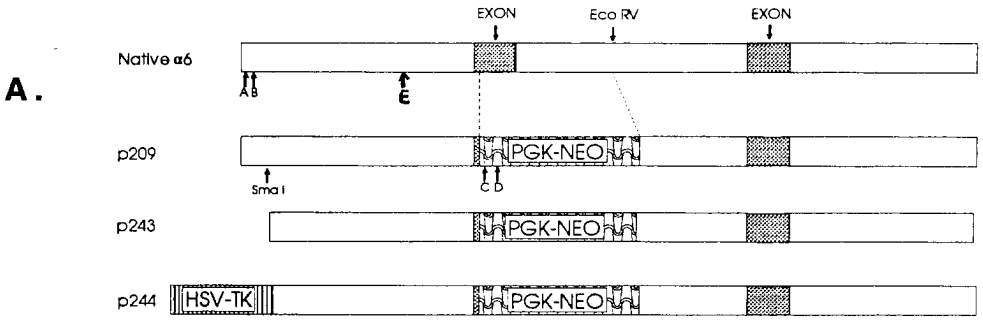
APPENDIX

1. Salary Support

The salary of Susan Domanico was supported by this grant at a rate of $26,200.00.

2. An abstract was presented at the "Fibronectin, Integrins, and Related Molecules" Gordon Conference, February 26-March 3, 1995.



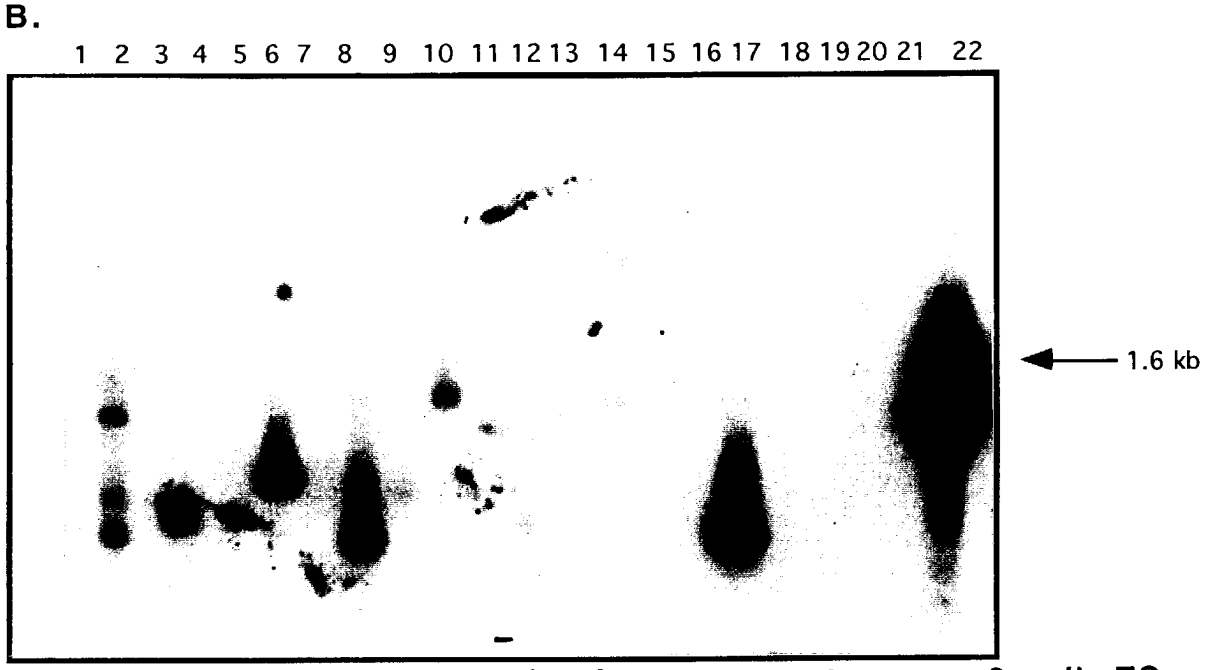


Fig 1. Homologous recombination to create an α6-null ES cell. A. The targeting vector is composed of a neomycin gene, driven by the mouse phosphoglucokinase (PGK) promoter. The neo-cassette replaces a 1.2 kb *Eco* RV fragment of α6 sequence, deleting the coding region for nucleotides 540-807 and 2/3 of the subsequent intron. The neo-cassette is inserted in the reverse orientation and is flanked by 2 kb and 1.8 kb of sequence homologous to the α6 gene. A *Sma* I site was engineered by mutating an A to G in plasmid p209. 200 bp 5' to the *Sma* I site were deleted to create plasmid p243. This enables PCR primers to be synthesized corresponding to the deleted α6 sequence. p243 has been transfected into ES1 cells and the cell line isolated, clone B5, serves as a control for the sensitivity of PCR. Clone B5 has been shown to carry only one copy of p243, as detected by Southern blot. p244 is the vector used for homologous recombination. B. PCR products generated from 19 pools of transfectants using oligos B and C were transferred to nylon and probed with ^{32}P end labelled oligo E. Lane 22 represents the PCR product from the positive control cell line, clone B5. The arrow marks the anticipated 1.6 kb band. Several "faint" false positives are detectable in lanes 10 and 14. These proved to be negative at the single clone level.

ES-1 ADHESION TO EHS LAMININ AND FIBRONECTIN

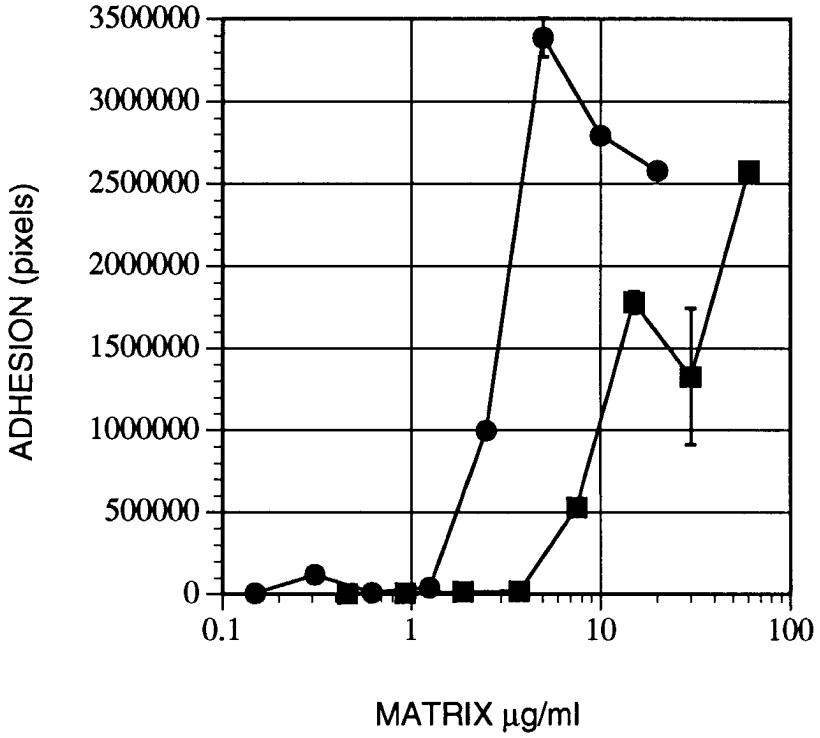


Fig 2. ES-1 cells adhere to EHS laminin and fibronectin. EHS laminin or bovine fibronectin, diluted in Hanks buffered saline solution (HBSS) were coated for 4 hr at 4°C. Matrix was removed and wells were blocked with 2% heat denatured BSA in HBSS, overnight at 4°C. ES-1 cells were labelled overnight with ^{35}S-methionine, harvested and plated at $5x10^4$ cells/well. Plates were incubated 30 min at 37°C, inverted, centrifuged at 800 rpm, for 8 min. Unbound cells were washed by immersion in ice cold PBS. Bound cells were fixed and radioactivity was measured using a Molecular Dynamics phosphorimager. Shown are an average of triplicate points.

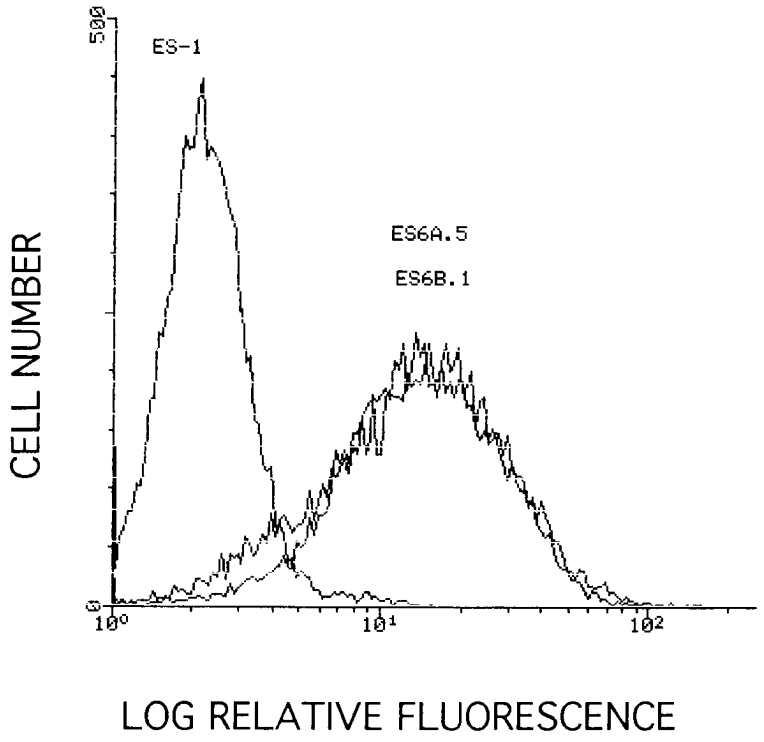


Fig 3. ES6A.5 and ES6B.1 express similar levels fo human α6. Cells were prepared for flow cytometry as described {682}. Anti-human α6 monoclonal antibody, BQ16, was used as a 1:5000 dilution of ascites. Goat anti-mouse IgG-FITC (Sigma) was used at a dilution of 1:200. Cells were analyzedon a Becton Dickenson FACScan. ES-1 cells were stained at the negative control. ES6A.5 and ES6B.1 bind comparable amounts of BQ16 antibody.

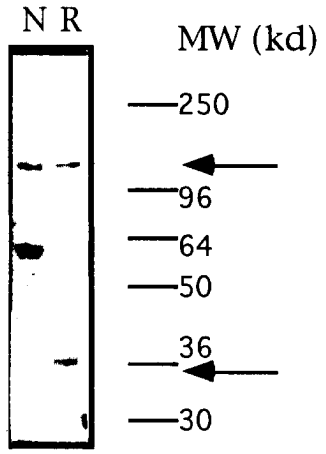


Fig 4. Western analysis of ES6A.5 expression of human α6A. Detergent lysate of ES6A.5 was separated on a 4-20% SDS-PAGE gradient gel. Samples were electrophoresed under not-reducing (N) and reducing (R) conditions. Proteins were transferred to PVDF using a semi-dry transfer cell. Human α6A was detected using affinity purified 6845 at 3.7 μg/ml. Antibody was visualized using donkey anti-rabbit-horseradish peroxidase (Amersham) and development with ECL chemiluminescence (Amersham). Molecular weight standards are marked on the right and the unreduced heavy chain (H) and reduced light chain (L) of α6 are indicated with arrows.

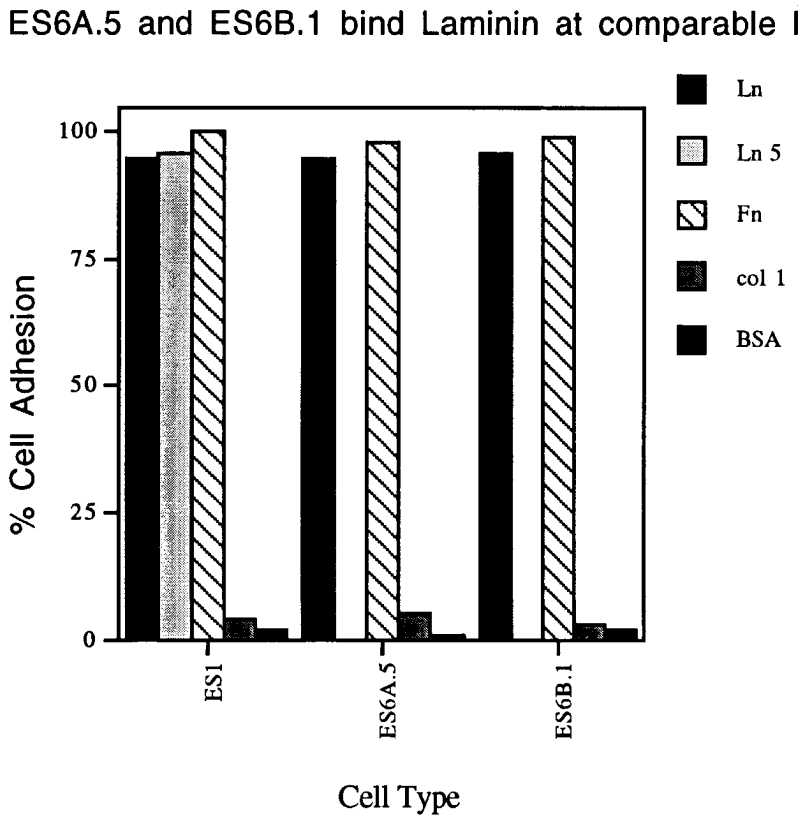


Fig 5. ES6A.5 and ES6B.1 cells bind EHS laminin at comparable levels. EHS laminin, bovine fibronectin, and rat collagen were diluted to 40 μg/ml in Hanks buffered saline solution (HBSS) and coated on to polystyrene for 4 hr at 4°C. Matrix was removed and wells were blocked with 2% heat denatured BSA in HBSS, overnight at 4°C. ES-1, ES6A.5 and ES6B.1 cells were labelled overnight with ^{35}S-methionine, harvested and plated at $5x10^4$ cells/well. Plates were incubated 30 min at 37°C, inverted, centrifuged at 800 rpm, for 8 min. Unbound cells were washed by immersion in ice cold PBS. Bound cells were fixed and radioactivity was measured using a Molecular Dynamics phosphorimager. Shown are an average of triplicate points.

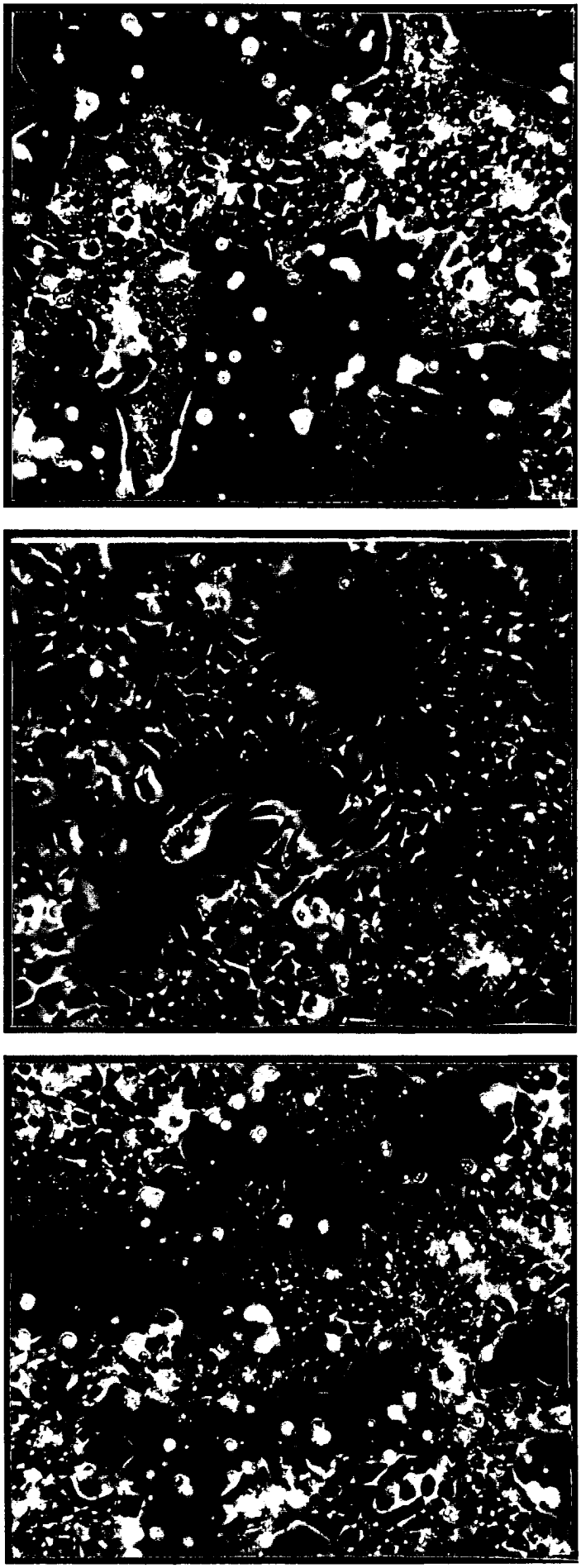


Fig 6. ES6A.5 cells have a different morphology than ES-1 and ES6B.1 cells. ES-1 (top panel), ES6A.5 (middle panel), and ES6B.1 (bottom panel) were grown on 0.1% gelatin until confluency. Shown are representative fields of each cell line. Photographs were taken on an Olympus phase contrast microscope at 20x magnification. The ES-1 and ES6B.1 cells grew in smooth-edged islands, characteristic of ES cells, while the ES6A.5 cells grew in looser islands, exhibited more jagged edges, and extened more processes.

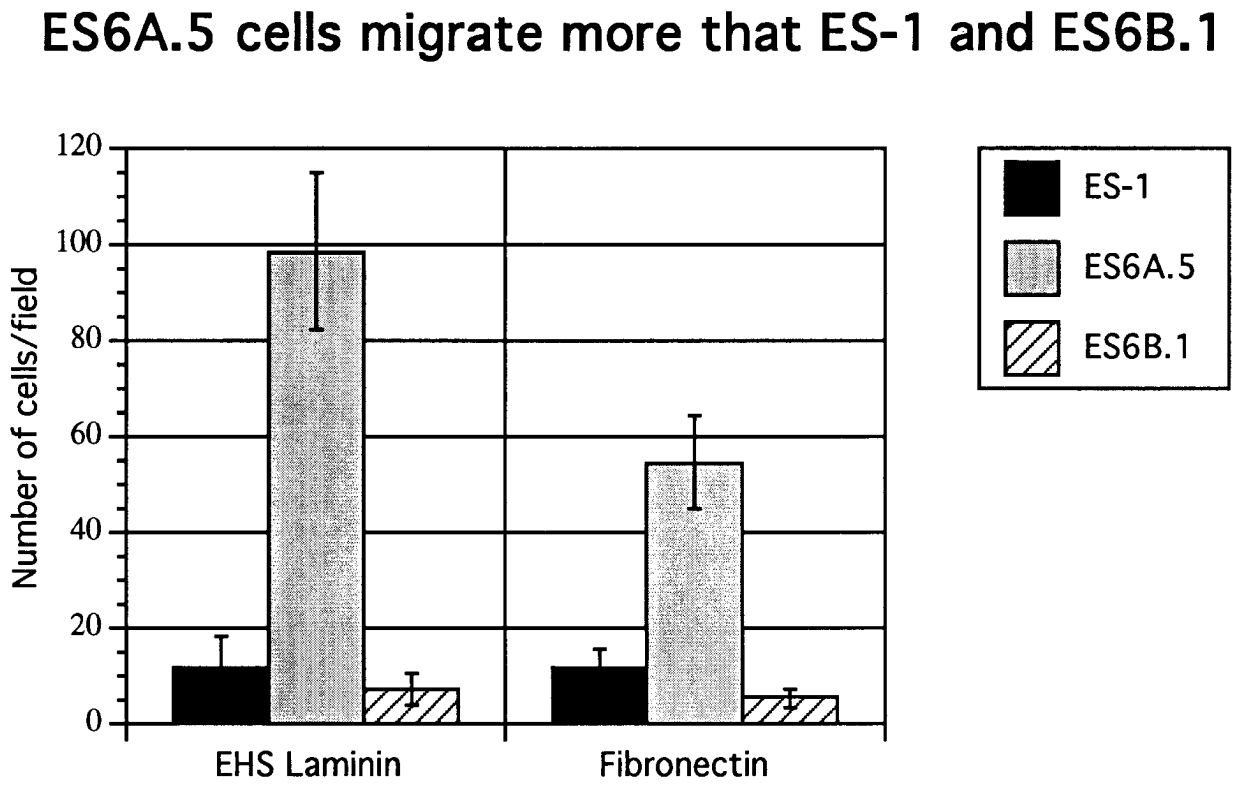


Fig 7. ES6A.5 cells are more migratory than ES-1 or ES6B.1 cells. Transwell membranes were coated with 40 μg/ml EHS laminin or bovine fibronectin in HBSS for 4 hr at 37°C. Matrix was removed by aspiration, and $6x10^4$ cells in 0.3 ml DMEM + sodium pyruvate, glutamine and LIF (C-DMEM) were added to the top chamber. C-DMEM (0.5ml was added to the bottom chamber. Cells were incubated 18 hr at 37°C. Media was aspirated, and the cells on the membranes were fixed and stained. The top of the membranes were swabbed with a cotton applicator to remove cells that did not migrate through the membrane. Membranes were cut out, mounted and cells were counted using a 40x objective. All cells within a field were counted.

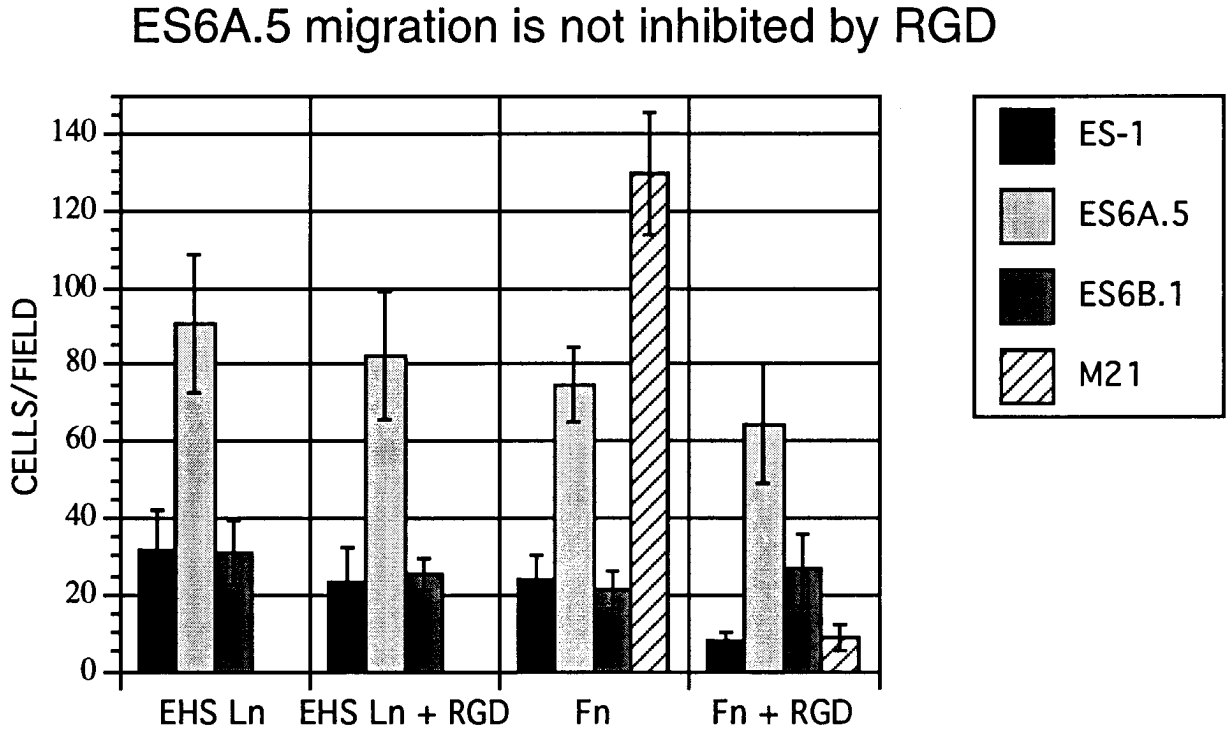


Fig 8. ES6A.5 cell migration on fibronectin is not RGD dependent. Transwell membranes were coated with 40 μg/ml EHS laminin or bovine fibronectin in HBSS for 4 hr at 37°C. Matrix was removed by aspiration, and $6x10^4$ cells in C-DMEM with or without 268 μM RGD peptide were added to the top chamber. C-DMEM with or without 268 μM RGD peptide (0.5ml) was added to the bottom chamber. Cells were incubated 18 hr at 37°C. Media was aspirated, and the cells on the membranes were fixed and stained. The top of the membranes were swabbed with a cotton applicator to remove cells that did not migrate through the membrane. Membranes were cut out, mounted and cells were counted using a 40x objective. All cells within a field were counted. As control for RGD inhibition, M21 cells did not migrate on fibronectin in the presence of RGD. There was no effect of RGD on the migration of ES6A.5 cells.

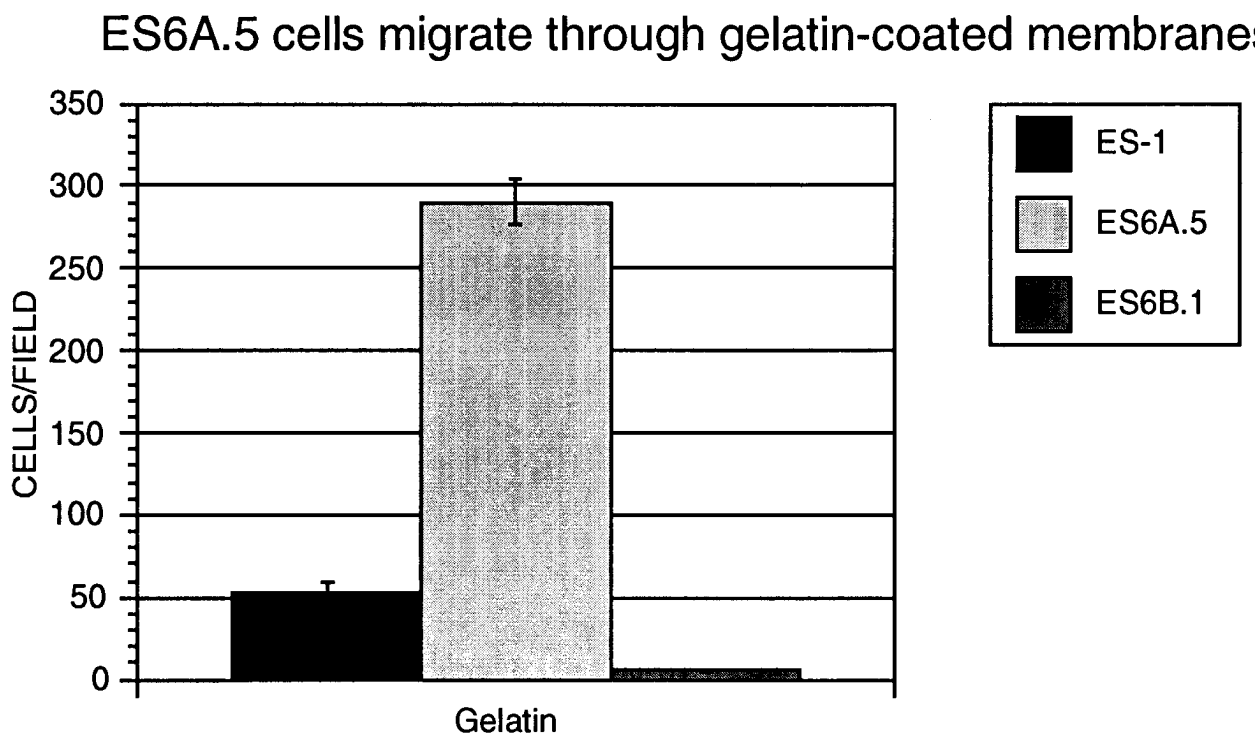


Fig 9. ES6A.5 cells migrate through gelatin-coated Transwell membranes. Transwell membranes were coated with 0.1% denatured gelatin for 4 hr at 37°C. Gelatin was removed by aspiration, and $6x10^4$ cells in 0.3 ml DMEM + sodium pyruvate, glutamine and LIF (C-DMEM) were added to the top chamber. C-DMEM (0.5ml was added to the bottom chamber. Cells were incubated 18 hr at 37°C. Media was aspirated, and the cells on the membranes were fixed and stained. The top of the membranes were swabbed with a cotton applicator to remove cells that did not migrate through the membrane. Membranes were cut out, mounted and cells were counted using a 40x objective. All cells within a field were counted. ES6A.5 cells migrated through the Transwell membrane, while ES-1 and ES6B.1 cells did not.